Antimicrobial with Time-kill Kinetics, Antioxidant, and Anticancer Properties of *Rosmarinus officinalis* L. Oil Extract Based on Its Bioactive Components

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There is a major clinical problem associated with antimicrobial resistance. *Rosmarinus officinalis* L. is an effective medicinal source. Its oil has been extracted and tested for its multiple therapeutic capabilities. The oil extract was found to be a broad-spectrum antimicrobial agent against *Bacillus subtilis* and *Staphylococcus aureus* as Gram-positive bacteria, *Escherichia coli* and *Klebsiella pneumonia* as Gram-negative bacteria, and *Candida albicans* as the most common pathogenic mold. The minimum inhibitory concentration of the oil extract was found to be 15.6 µg/mL against *B. subtilis*, *S. aureus*, and *C. albicans*, and 62.5 µg/mL and 125 µg/mL against *E. coli* and *K. pneumonia*, respectively. The bactericidal activity started at 150 and 180 min against Gram-positive and Gram-negative bacteria, respectively, with clear time-killing kinetics. The oil extract was able to scavenge DPPH free radicals with an IC_{50} of 4.0 µg/mL. The oil extract was found to have high toxicity on the Caco2 cell line (colon tissue) at high dose 1000 µg/mL with IC_{50} of 75.39 ± 0.56 µg/mL. The chemical composition of the oil extract was determined employing gas chromatography/mass spectrometry, in which 53 compounds were named at different surface area ratios, retention times, and probability ratios.

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INTRODUCTION

The medical field, especially the clinical one, has long been plagued by a major problem known as multidrug resistance, which is the ability of some microbes to resist different antimicrobial agents (Uddin *et al*. 2021; Alsolami *et al*. 2023; Saied *et al*. 2023).

There is a modern term called superbug microbes, which are resistant to different antibiotics at very high concentrations even above the approved minimum inhibitory concentrations (MICs), causing serious medical conditions. Numerous synthetic and semi-synthetic antibiotics are widely used worldwide to treat serious indications, but they have many side effects that emerge in some patients, especially if administered for a long time. Hence, the general medical trend resorts to using natural antibiotics extracted from microorganisms and plants, especially those with therapeutic properties called medicinal plants as studied by Almehayawi *et al.* (2024) and Selim *et al*. (2024). Although an extensive variety of therapeutic plants are known and widely exploited for this function as mentioned by Yahya *et al*. (2022) and Qanash *et al.* (2024), rosemary (*Rosmarinus officinalis* L.) still ranks first in use, because it is available, affordable, easy to grow, and has effective therapeutic properties (Belbachir *et al.* 2025).

Rosmarinus officinalis L. contains potent therapeutic essential oils (EOs) that include antibacterial agents with different mechanisms. Therefore, these EOs are widely employed in foodstuff preservation and pharmaceutical industry as an alternative medicine (Bakri *et al*. 2024). The *R. officinalis* extracts are rich in antibacterial volatile oils, including camphor, and α-pinene, besides phenols, including acids of rosmarinic and carnosic (Yeddes *et al*. 2021). *R. officinalis* is a perennial shrub and has many aromatic and medicinal components (Rafie and Soheila 2017). The leaves of *R. officinalis* are described as an ancient remedy, as they were consumed by the ancient Egyptians and Greeks to stop various diseases and were also used as preservatives and food flavorings (Couto *et al.* 2012). The *R. officinalis* extracts contain various antioxidant compounds, which play important roles, including preventing food decomposition and delaying diseases and aging. Antioxidants inhibit oxidation by preventing the initiation of oxidative chain reactions. The most common antioxidants rich in *R. officinalis* are phenols (flavonoids, tannins, hydroxycinnamate esters, and lignin), vitamin C, vitamin E, and glutathione, which limit the spread of reactive oxygen species (Khojasteh *et al.* 2020). The Lamiaceae family includes a wide range of medicinal plants, especially *R. officinalis*, which is rich in various therapeutic components, including powerful antioxidants, like caffeic acid, carnosic acid, and rosmarinic acid as polyphenolic compounds (Singh and Manna 2022).

The *R. officinalis* L. species is widely distributed throughout the world even in harsh environments, such as saline soils, so it has priority for use as a research material. Egypt is rich in *R. officinalis,* especially at the Mediterranean coast. Therefore, the Bedouin inhabitants of these coastal areas, who have inherited herbal medicine, use *R. officinalis* in many cases that require quick and effective treatment. Therefore, *R. officinalis* is widely used due to its antimicrobial, antioxidant, anti-inflammatory, antidiabetic, anticoagulant, anticancer, anti-obesity, dietary supplement, and hepatoprotective effects. Moreover, *R. officinalis* is used as an antispasmodic for renal colic and dysmenorrhea, a bronchodilator, a cough suppressant, an expectorant, a mucolytic, and a hair growth stimulant. Phenolic compounds have the upper hand in providing all of the above therapeutic properties to *R. officinalis*, and therefore, it is considered the dark horse in the treatment of many severe indications (Fernández-Ochoa *et al.* 2017; Nieto *et al*. 2018).

Rosmarinus officinalis has important medicinal uses, due to its ability to induce apoptosis of various cancer cell lines, including breast, colon, liver, and leukemia (Rafie *et al*. 2017). As reported in several studies, carnosic, carnosol, and ursolic acids showed strong toxicity against cancer cells even at advanced levels leading to complete cure (González-Vallinas *et al*. 2015; Chan *et al*. 2021). The anticancer activity of *R. officinalis*

acts as a mediator in numerous mechanisms, comprising apoptosis, arrest of cell cycle, delay of angiogenesis, and alteration of signaling pathways required for cell division. Cancer cell apoptosis is induced by carnosic acid and carnosol, which activate caspase cascades, disrupt the mitochondrial membrane, and regulate the proteins design of pro- and anti-apoptotic. Other components present in *R. officinalis* extracts kill cancer cells by inhibiting cancer-promoting enzymes, including matrix metalloproteinase, which are involved in tumor invasion and metastasis (Bozin *et al.* 2007). As mentioned in the current introduction, there are many articles available on *R. officinalis* L. but the novelty in the present investigation focused on antimicrobial activity and studying the Time-kill Kinetics and its anticancer activity against cancer cells associated with the digestive system. Therefore our research paper aims to show the therapeutic potential of different components present in *R. officinalis* L. oil extract, as well as to recognize the chemical constitution of the ethanolic extract of the oil using the GC-MS technique.

EXPERIMENTAL

Oil Extraction from *R. officinalis* L. Plant

Fresh *R. officinalis* L. plant (100 g) was ground in a mortar to obtain a fine paste, then placed in a flask containing distilled water (1000 mL), followed by the addition of 200 mL of chloroform to obtain organic phase. The extraction time continued to 4 h. By means of a rotating evaporator (Buchi Rotavapor E-210, China), the solvent was removed at 25 °C. The concentrated extract was washed by 4 mL of ethyl alcohol and then transferred to an ampoule. *Via* a slight stream of 1 L/min nitrogen, the residual of ethanol was then evaporated and the extract was reconstituted in 2 mL of ethyl alcohol (Teixeira *et al.* 2007).

Dilution of Antimicrobial Agent

Dilution was completed utilizing the broth microdilution approach illustrated by Wiegand *et al.* (2008), where small volumes of broth medium were dispensed into sterile plastic microdilution trays, hence the name. Microdilution trays consist of 96 wells, and all wells were filled separately with broth (0.1 mL) using a fractionator in which a twofold medium of antimicrobial agent was volumetrically diluted into the broth.

Preparation and Storage of Antimicrobial Agent

The microbial inoculum was prepared by the direct suspension of colony technique. Sterile saline solution was inoculated with bacterial colonies grown on blood agar for 18 to 24 h, then incubated for 24 h. Turbid growth was adjusted to meet the 0.5 McFarland standard using colorimetry, where 1 to 2×10^8 CFU/mL was obtained. Within 15 min, the bacterial suspension was diluted using sterile distilled water to obtain an inoculum of 2 to 8×10^5 CFU/mL, and it was optimized at 5×10^5 CFU/mL. Within 15 min, each well in the microdilution tray was inoculated with a bacterial inoculum (inoculum volume not exceeding 10% of the well volume). The inoculated microdilution trays were placed in plastic bags and sealed with plastic tape to avoid drying out and then incubated at 35 ± 2 °C for 16 to 20 h in an ambient air incubator at different distances and heights to avoid stacking to maintain the same incubation temperature for all bacterial cultures (Alghonaim *et al.* 2024).

Estimation of Minimal inhibitory Concentration (MIC)

A pure culture of the test bacterium was inoculated into trypsinized soy broth and incubated overnight. The bacterial suspension was diluted in trypsinized soy broth to obtain 1×10^5 to 1×10^6 CFU/mL. The antimicrobial agent stock was diluted to approximately 100 times the MIC limit. A stock solution of the extract containing 1000 µg/mL was diluted to obtain 500 µg/mL, and then diluted in a serial manner to obtain 1.95 µg/mL. Each dose was injected in the inoculated 96-well microtiter plate by cultured trypsinized soy broth with microorganisms. The negative control was twofold diluted (1000 – 1.95 µg/mL) free of microorganism inoculum. The positive control was the bacterial suspension grown in the same broth free of antimicrobial agent under the same conditions. The microtiter plates were incubated at 35 ± 2 °C for 16 to 20 h. The absorbance of the turbid growth was measured at 630 nm using a Bio-Tek 800 TS microplate reader. Bacterial growth was tested by colorimetry and compared in broth with and without antimicrobial agent, and the growth endpoint or MIC was precisely determined (Al-Rajhi *et al*. 2024).

Estimation of Minimal Bactericidal Concentration (MBC)

The MBC was determined based on the MIC dilution and the two most concentrated dilutions of the antimicrobial agent, which were plated and numbered to determine the viable CFU/mL. On Mueller Hinton agar plates enriched with 10% sheep blood, 100 mL of microbial culture was grown from each well that shown full inhibition of growth, from the final positive, and from the growth control to get the MBC. The plates underwent a 72-h microaerophilic incubation period at 35 °C. To ascertain the cidal or static impact of the examined extracts on microorganisms, the MBC/MIC ratios were computed (French 2006).

Antimicrobial Activity Determination

Antimicrobial effect was operated by the agar well manner *versus Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Klebsiella pneumonia* ATCC 13883, *Candida albicans* ATCC 10221, and *Aspergillus brasiliensis* ATCC 16888. Nutrient agar/potato dextrose agar was operated for bacteria/fungi inoculation with the best inoculum size for the test microorganisms, then transferred into plates, which are called seeded plates. The agar surface was cut using a sterile corkborer to make wells (6 mm in diameter). Each well was filled with 100 µL of oil extract compared to those loaded with 100 µL of standard antibiotic (1.0 mg/mL). Agar plates were incubated at 37 ℃ for 24 h and at 25 ℃ for 72 h with bacteria and fungi, respectively. The inhibition zones that appeared around the wells were used to indicate antimicrobial activity, and *vice versa* (Qanash *et al*. 2022).

Time-kill Kinetics Test

This test was used to identify the type of antimicrobial activity (bacteriostatic or bactericidal) of the oil extract of *R. officinalis* L. against Gram-positive and Gram-negative bacteria over time. In bactericidal activity, more than 99.9% was killed, which is equivalent to 3 $\log_{10}$ times the colony forming units (CFU) of the bacterial sample in a given time. Nutrient broth was inoculated with a test bacterial strain and incubated at 37 ℃ for 24 h. Bacterial suspension was supplemented with the oil extract at MIC. Nutrient broth without inoculations was considered as a positive control, while nutrient broth inoculated with a reference bacterial strain was considered as a negative control. The CFU/mL log was set at zero, 30, 60, 120, 150, and 180 min (Chinedu *et al*. 2024).

Evaluation of Antioxidant Activity

Antioxidant action was evaluated by measuring DPPH **(**Abdelghany *et al*. 2019**)**. Three milliliters of test material at varying concentrations (1.95, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL) were combined with 1.0 milliliter of the DPPH solution (0.1 mM) and ethanol. After giving the mixture a good shake, it was allowed to sit at room temperature for half an hour. A Milton Roy UV-VIS spectrophotometer (Nicolet evolution 100, Cambridge, MA, USA) was applied to notice absorbance (517 nm). The investigation was carried out employing ascorbic acid as a standard. The log dose-prevention curve was applied to decide the IC_{50}. Higher free radical potential was indicated by means of the reaction mixture's low absorbance. The DPPH scavenging effect percentage was calculated using Eq. 1:

$$\text{DPPH scavenging (\%)} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs.control}} \times 100 \quad (1)$$

Determination of Cytotoxicity of Test Sample

Wells containing tissue culture medium in the plate were inoculated with 1×10^5 CFU/mL and well-kept at 37 °C up to one day. The growth medium was harvested from the wells after the appearance of a monolayer, and then it was washed twice through water. Double dilution was performed in RPMI medium twice in the presence of 2% serum. Each dilution fraction (0.1 mL) was analyzed in all wells except 3 wells (control). Then the cytotoxicity was tested to detect abnormalities compared to controls (Qanash *et al*. 2023). The wells were supplemented with 20 µL of MTT solution and incubated in a under shaking at 37 °C and 150 rpm for 5 min, then the period of incubation completed at 37 °C for 4 h and at 5% CO_2 The medium was disposed of, and the plate was dried by sterile tissue to eliminate impurities. The reaction product was supplemented with 200 µL of DMSO and incubated under shaking at 37 °C and 150 rpm for 5 min, and then at 560 nm, the absorbance was deliberate.

GC-MS Analysis

The chemical alignment of the sample was determined operating a Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) through a TG–5MS direct capillary column (film thickness 30 m × 0.25 mm × 0.25 µm). The column oven temperature was held at 35 °C and then extended 3 °C/min to 200 °C for 3 min and then increased to the final temperature of 280 °C by 3 °C/min and held for 10 min. The temperature of the injector and MS transfer line was maintained at 250 and 260 °C, respectively. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The solvent delay was 3 min and 1 µL diluted samples were automatically injected using the Autosampler AS1300 coupled to the GC in split mode. The EI mass spectra were collected at an ionization voltage of 70 eV over the range of 40 to 1000 *m*/*z* in full scan mode. The ion source temperature was set at 200 °C. The components were identified by comparing their retention times and mass spectra with those in the WILEY 09 and NIST 11 mass spectra databases (Al-Rajhi and Abdelghany 2023).

Statistical Analysis

The findings were demonstrated as mean ± standard deviation (SD) and mean ± standard error (SE), which were computed with SPSS v.25 and Microsoft Excel 365. One-

way investigation of variance (ANOVA) was applied to assess quantitative data with a standard distribution across various treatments at a 0.05 possibility level.

RESULTS and DISCUSSION

The oil extract of *R. officinalis* L. was tested for its antimicrobial ability *versus* fungi and versus bacteria belonging to both Gram-positive and negative (Table 1 and Fig. 1). The oil extract activity against microorganisms was compared with 1.0 mg/mL of gentamycin and fluconazole as standard antibacterial and antifungal antibiotics, respectively. The oil extract showed broad-spectrum antimicrobial action, affecting *B. subtilis*, *S. aureus*, *E. coli, K. pneumonia,* and *C. albicans* but not *A. brasiliensis*. Gram-positive bacteria were most affected by the oil extract, followed by *C. albicans* and bacteria of Gram-negative. Naturally, the oil extract showed less activity against bacteria development of Gram-negative, due to their different line of defense and resistance mechanisms to antimicrobial agents. The oil extract showed inhibition regions of 28 ± 0.1 and 31 ± 0.1 mm compared to gentamycin activity of 27 ± 0.2 and 26 ± 0.2 mm for *B. subtilis* and *S. aureus*, correspondingly. Therefore, the oil extract was more effective than gentamycin, and *S. aureus* was more sensitive to the activity of the oil extract than *B. subtilis*. The oil extract showed inhibition zones of 23 ± 0.1 mm and 20 ± 0.2 mm compared to gentamycin activity of 25 ± 0.1 mm and 20 ± 0.1 mm for *E. coli* and *K. pneumonia*, respectively. Therefore, the oil extract was less effective than gentamycin, and *E. coli* was more susceptible to the activity of the oil extract than *K. pneumonia*. The oil extract revealed an inhibition area of 30 ± 0.1 mm compared to fluconazole activity of 31 ± 0.1 mm for *C. albicans*. Therefore, the oil extract was less effective than fluconazole. The oil extract did not show antifungal activity against *A. brasiliensis* compared to fluconazole activity of 25 ± 0.2 mm. This may be because of cell wall structure where it is different from the wall structure of bacteria. Yeddes *et al*. (2022) informed that rosemary essential oil showed bactericidal activity against bacteria belonging to Gram-negative, including *Campylobacter jejuni*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Escherichia coli*, besides Gram-positive, including *Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Bosnić *et al*. (2006) informed that rosemary (Bosnian origin) essential oil exhibited prevention action for growth of *E. coli* and *P. aeruginosa* as Gram-negative bacteria, as the essential oil was found to contain high levels of 1,8-cineole (Kifer *et al*. 2016), camphor (Zainudin and Mohd 2015), and α-pinene (Ngân *et al*. 2019).

Table 1. Antimicrobial Activity of Volatile Oil Extract of *R. officinalis* L. Against Pathogens

Pathogens	Mean of Antimicrobial Activity by Inhibition Zone (mm)		
	Oil Extract	Standard Antibiotics (1 mg/mL)	
		Antibacterial (Gentamycin)	Antifungal (Fluconazole)
B. subtilis	28 ± 0.1	27 ± 0.2	----------
S. aureus	31 ± 0.1	26 ± 0.2	----------
E. coli	23 ± 0.1	25 ± 0.1	----------
K. pneumonia	20 ± 0.2	20 ± 0.1	----------
C. albicans	30 ± 0.1	----------	31 ± 0.1
A. brasiliensis	NA	----------	25 ± 0.2

NA: No activity

The oil extract showed different MICs against different pathogens (Table 2). The oil extract showed MIC of 15.6 µg/mL against *B. subtilis*, *S. aureus,* and *C. albicans*. The oil extract showed MICs of 62.5 and 125 µg/mL toward *E. coli* and *K. pneumonia*, respectively. The oil extract showed different MBCs against different pathogens (Table 3). The extract of oil showed MBC of 31.2 µg/mL toward *B. subtilis* and *S. aureus*. The extract of oil showed MBCs of 125 µg/mL and 500 µg/mL against *E. coli* and *K. pneumonia*, respectively. The oil extract showed MBC of 62.5 µg/mL versus *C. albicans*. Aseer *et al*. (2021) registered that the leaves extract via ethanol of rosemary presented potent activity against growth of clinical isolates, including *S. aureus*, *Enterococcus* sp., *Streptococcus pyogenes*, *Salmonella* sp., *Shigella* sp., *Klebsiella pneumoniae*, *P. aeruginosa*, *E. coli*, *Proteus* sp., and *Campylobacter* sp. Furthermore, the MIC of the ethanolic extract of rosemary at dose from 4×10^3 µg/mL to 32×10^3 µg/mL with MBC at dose from 8×10^3 µg/mL to 32×10^3 µg/mL. The MIC and MBC were shown toward *S. aureus*, while their top quantities were shown against *E. coli*.

The oil extract showed antibacterial activity with different time-kill kinetics over time ranging from zero to 180 min (Table 3). In Gram-positive bacteria, bactericidal activity was found to start at 150 min, while in other groups of bacteria (Gram-negative) it was found to start at 180 min. The oil extract revealed superior antibacterial potential toward Gram-positive if compared to Gram-negative group. At zero time, the bacterial counts were found to be $112 \times 10^5 \pm 2.0$ CFU/mL, $55 \times 10^5 \pm 1.0$ CFU/mL, $17 \times 10^5 \pm 2.0$ CFU/mL, and $21 \times 10^5 \pm 2.0$ CFU/mL with *B. subtilis*, *S. aureus*, *E. coli*, and *K. pneumonia*, respectively. The count of bacteria gradually decreased over time until they were completely absent at 150 min for *B. subtilis* and *S. aureus*, and at 180 min for *E. coli* and *K. pneumonia*.

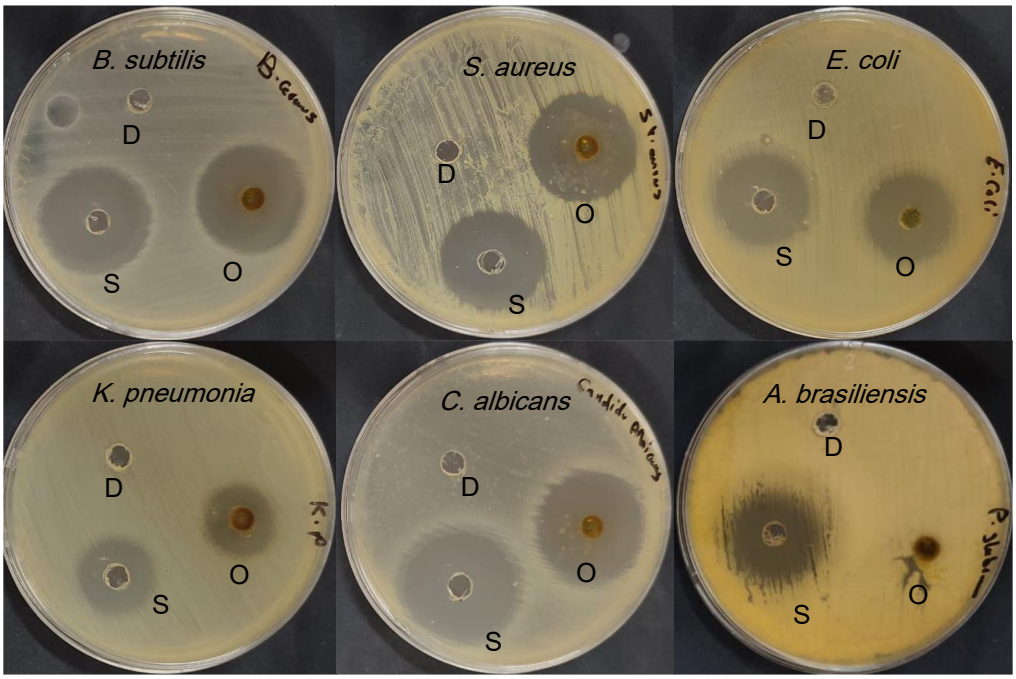

Fig. 1. Antimicrobial activity of oil extract of *R. officinalis* L. (O) and standard (Gentamycin / Fluconazole) (S), and DMSO (D) against pathogens

Table 2. Determination of MIC and MBC (µg/mL) of Volatile Oil Extract of *R. officinalis* L. Against Pathogens

Concentration (µg/mL)	Pathogens									
	B. subtilis		*S. aureus*		*E. coli*		*K. pneumonia*		*C. albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1.95	+	+	+	+	+	+	+	+	+	+
3.90	+	+	+	+	+	+	+	+	+	+
7.81	+	+	+	+	+	+	+	+	+	+
15.62	-	+	-	+	+	+	+	+	-	+
31.25	-	-	-	-	+	+	+	+	-	+
62.5	-	-	-	-	-	+	+	+	-	-
125	-	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-	-
500	-	-	-	-	-	-	-	-	-	-
1000	-	-	-	-	-	-	-	-	-	-

+; The growth is present, -; The growth is absent (no growth)

Chinedu *et al*. (2024) reported that time-kill kinetics of ethanolic extract of *Allium sativum* at quantities of 0.5, 1.0, and 2 mg/mL were measured to repress *S. aureus* and *P. aeruginosa*. The ethanolic extract showed bacteriostatic activity at 0.25 mg/mL, while it showed bactericidal activity at 0.5 and 1 mg/mL versus *S. aureus* and *P. aeruginosa* after 24 and 12 h, correspondingly. A significant decline ($p \leq 0.05$) in the count of viable *P. aeruginosa* was observed over multiple time periods at 1.0 mg/mL at 0.09 log10 to 1.20 log10 after 10 h, and at 0.5 mg/mL at 0.02 log10 to 0.52 log10 after 12 h. The reduction in viable *P. aeruginosa* ranged from ≥ 18.35% to ≤ 99.9% at 1.0 mg/mL, and ≥ 5.30% to ≤ 99.9% at 0.5 mg/mL between 2 h and 24 h. A significant decrease ($p \leq 0.05$) in the count

of viable *S. aureus* was observed over multiple time periods at 1.0 mg/mL at 0.09 log10 to 0.97 log10 after 10 h, and at 0.5 mg/mL at 0.03 log10 to 0.47 log10 after 12 h. The reduction in viable *S. aureus* ranged from ≥ 18.44% to ≤ 99.9% at 1.0 mg/mL, and ≥ 6.0% to ≤ 99.9% at 0.5 mg/mL between 2 h and 24 h.

Table 3. Time-kill Kinetics Assay of Antibacterial Activity of Volatile Oil Extract of *R. officinalis* L.

Time-kill Kinetics (min)	Antibacterial Activity (CFU/mL)			
	B. subtilis	***S. aureus***	***E. coli***	***K. pneumonia***
0.0	$112 \times 10^5 \pm 2.0$	$55 \times 10^5 \pm 1.0$	$17 \times 10^5 \pm 2.0$	$21 \times 10^5 \pm 2.0$
30	$20 \times 10^4 \pm 1.0$	$33 \times 10^3 \pm 2.0$	$22 \times 10^3 \pm 2.0$	$118 \times 10^4 \pm 2.0$
60	$17 \times 10^3 \pm 1.0$	$24 \times 10^2 \pm 2.0$	440 ± 3.0	$38 \times 10^3 \pm 1.0$
120	110 ± 2.0	380 ± 2.0	156 ± 2.0	210 ± 2.0
150	0.0	0.0	12 ± 1.0	34 ± 2.0
180	0.0	0.0	0.0	0.0

Antioxidant activity was determined on the basis of prevention of lipid peroxidation, so scavenging DPPH free radicals. Standard ascorbic acid and oil extract of *R. officinalis* L. were prepared at different concentrations, which were experienced for their capability to scavenge DPPH by calculating its absorbance at 517 nm. The oil extract of *R. officinalis* L. presented antioxidant action by scavenging DPPH across IC_{50} of 4.0 µg/mL contrasted to the IC_{50} of ascorbic acid (standard) of 3.13 µg/mL (Table 4). Thus, there is a slight difference between the antioxidant activities of standard ascorbic acid and the oil extract of *R. officinalis* L., with the former showing a higher ability to scavenge DPPH. The DPPH scavenging rate started at its peak at 1000 µg/mL with standard ascorbic acid and *R. officinalis* L. oil extract, then gradually decreased with lower concentrations until it reached its lowest level at 1.95 µg/mL. Ahmad (2024) registered that the antioxidant potential of rosemary leaf extracts was tested in accordance with different components, including total contents of phenolic (TPC), flavonoid (TFC), and Tannin (TTC). Where the ethanolic extract showed maximum TPC (72.3 GAE mg/g) and TFC (26.8 RE mg/g), while the aqueous extract reflected TTA (20.2 GAE mg/g), and the methanolic extract reflected scavenging of free radicals NO (86.7 RE mg/g) and DPPH (138 GAE mg/g). Also, the aqueous extract reflected antioxidant activity in ABTS (125 TE mg/g), and ferric reducing power (144.5 AScE mg/g) compared to FRAP (130.5 AScE mg/g) and total antioxidant activity (179 GAE mg/g) utilizing the ethanolic extract of rosemary leaves. The outcomes of the paper of Ahmad (2024) exhibited that the highest coefficient of determination showing the link among the phytocomponents content and the antioxidant approaches used was among TPC, FRAP, and TFC.

Table 4. *In vitro* Assay of Antioxidant Activity of Oil Extract and Ascorbic Acid

Conc. (µg/mL)	Ascorbic Acid (Standard)				Oil Extract of *R. officinalis* L.			
	Mean A_{517}	% of DPPH Scavenging	± SD	± SE	Mean A_{517}	% of DPPH Scavenging	± SD	± SE
1000	0.027	98.2	0.004	0.001	0.038	97.5	0.002	0.001
500	0.057	96.2	0.003	0.001	0.071	95.3	0.002	0.001
250	0.096	93.7	0.002	0.000	0.105	93.0	0.004	0.001
125	0.140	90.7	0.002	0.001	0.159	89.5	0.003	0.001
62.5	0.257	82.9	0.002	0.001	0.271	82.0	0.004	0.001
31.25	0.397	73.7	0.003	0.001	0.440	70.8	0.005	0.001
15.625	0.519	65.5	0.003	0.001	0.538	64.3	0.005	0.001
7.8125	0.640	57.5	0.004	0.001	0.668	55.7	0.003	0.001
3.90	0.731	51.5	0.002	0.001	0.791	47.5	0.003	0.001
1.95	0.879	41.7	0.002	0.001	0.900	40.3	0.005	0.001
0.00	1.507	0.00	0.010	0.003	1.507	0.00	0.010	0.003
IC_{50}	3.13 µg/mL				4.0 µg/mL			

SD; Standard deviation, SE; Standard error

The oil extract was tested for its effect (toxicity/viability) on the Caco2 cell line (colon tissue) compared to a normal cell line that appeared free of abnormal signs. Different concentrations of rosemary extract were experienced for their influence on the cell line (Table 5). The oil extract presented the highest toxicity of 97.6% and the lowest viability of 2.41% at 1000 µg/mL. The cytotoxicity decreased and the viability increased with the slope trend of rosemary extract until the lowest cytotoxicity of 1.62% and the highest viability of 98.4% were reached at 31.2 µg/mL. Different concentrations of the oil extract showed cytotoxic and viability effect on colon tissues with IC_{50} of 75.39 ± 0.56 (Table 6). Additionally, morphological changes were observed on treated cancer cells to rosemary oil extract with clear apoptosis as cleared in Fig. 2, particularly at high concentrations. Eleni *et al*. (2022) reported that *R. officinalis* L. is extensively worked as a potent pharmaceutical plant due to its high cytotoxicity on different cell lines, including cancer cell lines of rhabdomyosarcoma and glioblastoma. The methanolic extract showed potent cytotoxicity based on time and dose on the two cell lines. The treated cell lines showed the lowest IC_{50} values at 72 h corresponding to 0.249 mg/mL for cell line of rhabdomyosarcoma and 0.577 mg/mL for cell line of glioblastoma.

Table 5. Cytotoxicity Effect of Rosemary on Caco2 Cell Line

Conc. (µg/mL)	Mean (A_{560})	± SE	Viability (%)	Toxicity (%)	IC_{50} ± SD (µg/mL)
0.0	0.72	0.002	100	0.0	75.39 ± 0.56
1000	0.017	0.0006	2.41	97.59	
500	0.021	0.0014	3.01	96.99	
250	0.028	0.0020	3.89	96.11	
125	0.083	0.0061	11.62	88.38	
62.5	0.339	0.0096	47.08	52.92	
31.25	0.708	0.0026	98.38	1.62	

SD; standard deviation, SE; standard error

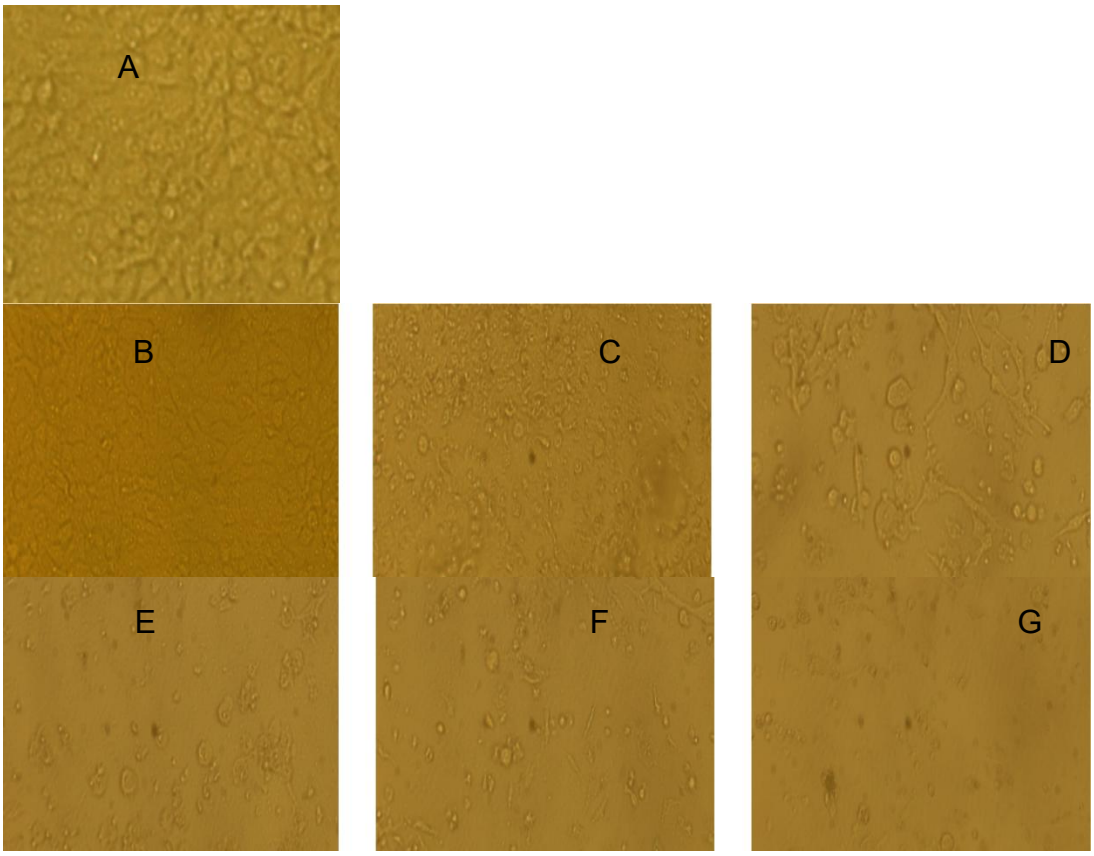

Fig. 2. (A) Caco2 cell line in colon tissues as a positive control; rosemary extract doses including (B) 31.25 µg/ mL, (C) 62.5 µg/ mL, (D) 125 µg/ mL, (E) 250 µg/ mL, (F) 500 µg/ mL, and (G) 1000 µg/mL

The oil extract was investigated using GC-MS and was noticed to contain 53 compounds at different retention times (RTs), surface area ratios, and probability ratios (Table 6), as shown in Fig. 3. The current findings showed that the RT increased towards peak 1 to peak 60, due to the increased association of the compound with the column as a stationary phase. Thus, the compound at peak 1 had the weakest association with the column, and then the association gradually increased until it reached the strongest state with the compound at peak 61. However, the boiling point and solubility of the compound in the stage of liquid, as well as the column temperature, affect the RT. Therefore, if the boiling point of the compound rises above the column temperature, the RT increases, and if the solubility of the compound in the liquid stage increases, the RT increases. Mehdi *et al*. (2011) reported that the ingredients of the essential oil of Iranian rosemary were identified through GC-MS analysis corresponding to 68 components. The analysis revealed that the most significant and influential components were 1,8-cineole (23.5%), α-pinene (21.7%), verbenone (7.57%), camphor (7.21%), and eucalyptol (4.49%).

Table 6. GC-MS Examination of Rosemary Extract

Peak	*RT (min)	% of Area	Constituent	**Prob (%)	Formula	***MW (g/mol)
1	11.17	0.29	O-Cymene	19.14	$C_{10}H_{14}$	134
2	11.42	6.44	Eucalyptol	46.20	$C_{10}H_{18}O$	154
3	11.51	3.65	Cyclohexanol,1-methyl-4-(1-methylethenyl)-acetate	16.31	$C_{12}H_{20}O_2$	196
4	14.51	0.70	1,6-Octadien-3-ol,3,7-dimethyl	57.98	$C_{10}H_{18}O$	154
5	15.53	0.38	Bicycloheptan-2-one,1,7,7-trimethyl-(1S)	25.34	$C_{10}H_{16}O$	152
6	15.61	0.70	Bicycloheptan-2-one,1,7,7-trimethyl- (1S)-	26.03	$C_{10}H_{16}O$	152
7	17.14	0.36	Bicycloheptan-2-ol,1,7,7-trimethyl (1S-endo)	21.90	$C_{10}H_{18}O$	154
8	17.35	0.73	*p*-Menthan-1-ol	11.86	$C_{10}H_{20}O$	156
9	17.51	0.81	*p*-Menthan-1-ol	12.08	$C_{10}H_{20}O$	156
10	17.68	7.05	Cyclohexanol,5-methyl-2-(1-methylethyl)	10.65	$C_{10}H_{20}O$	156
11	18.58	0.46	(+) Isopulegol	16.68	$C_{10}H_{18}O$	154
12	18.69	0.43	Ethyl linalool	36.63	$C_{11}H_{20}O$	168
13	19.28	1.37	Carbonic acid, but-3-yn-1-yl octyl ester	8.70	$C_{13}H_{22}O_3$	226
14	21.25	0.56	1,5-Dimethyl-1-vinyl-4-hexenyl 2-aminobenzoate	28.63	$C_{17}H_{23}NO_2$	273
15	22.65	0.36	Orthotertbutyl cyclohexyl acetate	37.80	$C_{12}H_{22}O_2$	198
16	26.48	0.42	2,6-Octadien-1-OL, 3,7-dimethyl- acetate	31.89	$C_{12}H_{20}O_2$	196
17	28.21	0.71	Caryophyllene	31.66	$C_{15}H_{24}$	204
18	30.36	0.50	ç-Ionone, methyl	33.52	$C_{14}H_{22}O$	206
19	31.65	0.42	1,4-Naphthalenedione, 4A,5,8,8A-tetrahydro-2-methoxy-4 A,8-dimethyl	29.21	$C_{13}H_{16}O_3$	220
20	32.10	0.31	Dodecanoic acid, 3-hydroxy	22.52	$C_{12}H_{24}O_3$	216
21	32.38	0.40	(-) Menthoxyacetyl chloride	5.28	$C_{12}H_{21}ClO_2$	232
22	34.31	3.62	Diethyl phthalate	80.26	$C_{12}H_{14}O_4$	222
23	35.84	0.47	Cedrol	13.43	$C_{15}H_{24}O$	220
24	36.59	4.05	Cyclopentane acetic acid, 3-oxo-2-pentyl-, methyl ester	92.56	$C_{13}H_{22}O_3$	226
25	36.85	0.97	Triethyl citrate	90.23	$C_{12}H_{20}O_7$	276
26	37.26	2.38	1-(4-Isopropylphenyl)-2-methylpropyl acetate	71.06	$C_{15}H_{22}O_2$	234
27	37.38	0.73	Cis-3-Hexenyl salicylate	80.74	$C_{13}H_{16}O_3$	220
28	37.48	0.99	1-(4-Isopropylphenyl)-2-methylpropyl acetate	35.16	$C_{15}H_{22}O_2$	234
29	38.35	0.46	1-(4-Isopropylphenyl)-2-methylpropyl acetate	19.07	$C_{15}H_{22}O_2$	234
30	42.37	1.51	5,5-Dimethyl-2-(7- hydroxy-N-heptyl)-2-N-hexyl-1,3-dioxane	19.07	$C_{19}H_{38}O_3$	314
31	42.56	0.86	9,12-Octadecadienoic acid	9.07	$C_{18}H_{32}O_2$	280
32	42.79	0.52	9,12-Octadecadienoic acid	14.92	$C_{18}H_{32}O_2$	280
33	43.63	9.45	5-Amino-2-(P-methoxymethyl)-2- methyl-2H-triazolo triazine	37.01	$C_{12}H_{14}N_6O$	258
34	43.94	3.20	Benzoic acid, 2-hydroxy-phenylmethyl ester	68.25	$C_{14}H_{12}O_3$	228
35	47.15	0.56	Musk ketone	40.89	$C_{14}H_{18}N_2O_5$	294
36	48.82	0.89	Hexadecanoic acid, ethyl ester	56.64	$C_{18}H_{36}O_2$	284
37	53.67	3.95	Linoleic acid ethyl ester	35.59	$C_{20}H_{36}O_2$	308
38	53.96	3.21	Ethyl oleate	17.80	$C_{20}H_{38}O_2$	310
39	55.03	0.53	Octadecanoic acid, ethyl ester	55.51	$C_{20}H_{40}O_2$	312

40	56.64	0.56	4,6-Bis (1,1'-dimethylethyl)-2',5'-dimethoxy-1, 1'-biphenyl-2-ol	36.62	$C_{22}H_{30}O_3$	342
41	57.79	1.17	3-Pyridinecarbonitrile,6-[2,2-Bis-(methylthio) ethenyl]-4-(4 -methoxyphenyl)-2-methyl	54.04	$C_{18}H_{18}N_2OS_2$	342
42	58.87	1.31	3-Pyridinecarbonitrile,2-ethoxy-5,6-dimethyl-4-(3,4,5-trimethoxyphenyl)	12.87	$C_{19}H_{22}N_2O_4$	342
43	59.45	0.49	3-Pyridinecarbonitrile, 6-[2,2-Bis-(methylthio) ethenyl]-4-(4 -methoxyphenyl)-2-methyl	10.07	$C_{18}H_{18}N_2OS_2$	342
44	60.19	0.91	Podocarpa-1,8,11, 13-tetraen-3-one, 14-isopropyl-1,13- dimethoxy	64.29	$C_{22}H_{30}O_3$	342
45	62.15	2.01	4,7-Methano-2H-inden-2-one, 4,5,6,7-tetrahydro -4,8,8-trimethyl-1 ,3-diphenyl	42.23	$C_{25}H_{24}O$	340
46	64.19	0.53	1,2-Propanediol, 3-(hexadecyloxy)-, diacetate	6.93	$C_{23}H_{44}O_5$	400
47	67.12	0.71	4H-1-Benzopyran -4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy	35.08	$C_{18}H_{16}O_7$	344
48	68.70	1.04	Cyclooctanepentanoic acid, 1-nitro-á,2-dioxo-, methyl ester	43.88	$C_{14}H_{21}NO_6$	299
49	68.90	0.73	2-Hydroxy-3-[(9E) -9-octadecenoyloxy] propyl (9E)-9-octadecenoate	24.80	$C_{39}H_{72}O_5$	620
50	69.68	0.57	9-Octadecenoic acid	9.26	$C_{18}H_{34}O_2$	282
51	71.78	3.44	13-Docosenamide	71.25	$C_{22}H_{43}NO$	337
52	73.25	0.88	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexa methyl	12.30	$C_{30}H_{50}$	410
53	75.44	1.04	Dotriacontane	29.96	$C_{32}H_{66}$	450
54	78.03	1.14	4,5-Epoxy-17-methyl-3-phthalimidomorphina N-6-ol	48.40	$C_{25}H_{24}N_2O_4$	416
55	79.32	9.39	(+)-Sesamin	61.77	$C_{20}H_{18}O_6$	354
56	79.79	1.92	Dotriacontane	25.70	$C_{32}H_{66}$	450
57	80.47	3.90	5,4-(Benzo-1,3-dioxol- 5-yl) hexahydrofuro-3, 4-C-furan-1-yl) oxy) benzo-1,3 dioxol	92.80	$C_{20}H_{18}O_7$	370
58	83.30	0.59	á-Sitosterol	56.88	$C_{29}H_{50}O$	414
59	83.93	1.54	Dotriacontane	30.53	$C_{32}H_{66}$	450

*RT; Retention time, **Prob; Probability, ***MW; Molecular weight

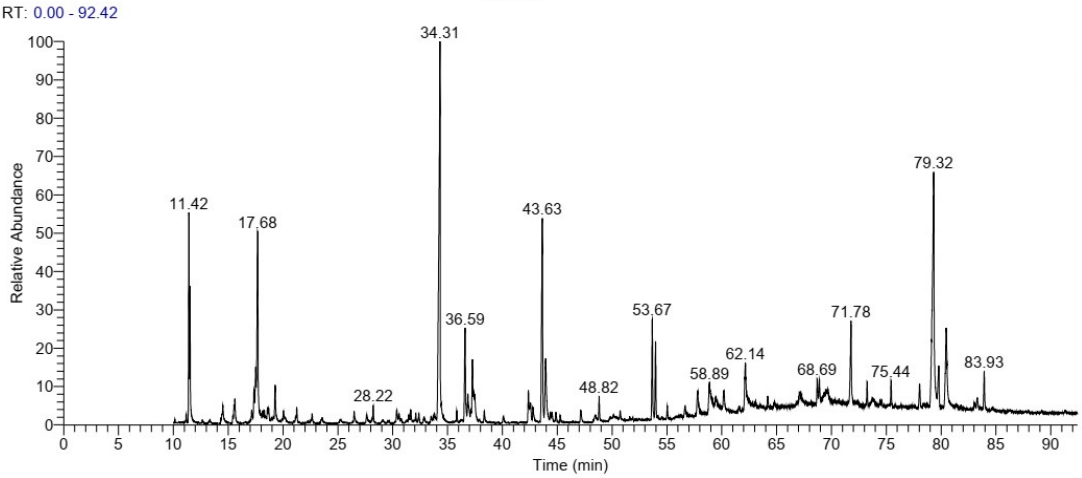

Fig. 3. Chromatogram from GC-MS analysis of rosemary extract

CONCLUSIONS

1. The present oil extract of was analyzed *via* GC-MS technique and found to be rich with numerous bioactive compounds, from which 5-amino-2-(P-methoxymethyl)-2-methyl-2H-triazolo triazine and (+)-sesamin were detected with the highest area 9.45 % and 9.39, respectively.
2. Oil extract of *Rosmarinus officinalis* L. possesses excellent antimicrobial (*C. albicans, B. subtilis*, *S. aureus, E. coli*, and *K. pneumonia*) and antioxidant (IC_{50} 4.0 µg/mL) activities.
3. Anticancer activity of *R. officinalis* L. extract was reported with IC_{50} of 75.39 ± 0.56 µg/mL against the Caco2 cell line.

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